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ORIGINAL ARTICLE

Reactive oxygen species elevation and recovery in Drosophila bodies and ovaries following short-term and long-term exposure to DECT base EMF

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Abstract

The objective of this study was to approach the basic mechanism(s) underlying reported ovarian apoptotic cell death and fecundity decrease induced by nonionizing radiation (NIR) in *Drosophila melanogaster*. ROS (Reactive Oxygen Species) levels were measured in the bodies and the ovaries of (sexually mature) 4-day-old flies, following exposure for 0.5, 1, 6, 24 and 96 h to a wireless DECT (Digital Enhanced Cordless Telephone) base radiation (1.88–1.90 GHz). Electrical field intensity was 2.7 V/m, measured within the fly vials and calculated SAR (Specific Absorption Rate) value = 0.009 W/Kg. Male and female bodies showed twofold increase in ROS levels ($p < 0.001$) after 6 h of exposure, slightly increasing with more irradiation (24 and 96 h). Ovaries of exposed females had a quick response in ROS increase after 0.5 h (1.5-fold, $p < 0.001$), reaching 2.5-fold after 1 h with no elevation thereafter at 6, 24 and 96 h. ROS levels returned to normal, in the male and the female bodies 24 h after 6 h of exposure of the flies ($p < 0.05$) and in the ovaries 4 h after 1 h exposure of the females ($p < 0.05$). It is postulated that the pulsed (at 100 Hz rate and 0.08 ms duration) idle state of the DECT base radiation is capable of inducing free radical formation albeit the very low SAR, leading rapidly to accumulation of ROS in a level-saturation manner under continuous exposure, or in a recovery manner after interruption of radiation, possibly due to activation of the antioxidant machinery of the organism.

Keywords

Drosophila melanogaster, electromagnetic fields, oogenesis, oxidative stress, reactive oxygen species, ROS recovery, wireless DECT, wireless DECT base

History

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Introduction

The last few decades a serious concern is expressed about the biological effects of the electromagnetic fields (EMFs) of nonionizing radiation (NIR), which are constantly growing with the development of telecommunication systems. Although the number of users and the amount of radio-frequency (RF) and microwave (MW) applications are expanding exponentially, along with the increase in relevant scientific papers, nevertheless the results from this research field remain still controversial. Predominant devices of everyday use include the mobile phones and the wireless DECT (Digital Enhanced (European) Cordless Telephones) at home and at work. To ensure the smooth operation of the system, a DECT base device transmits signals continuously (Figure 1) to enable synchronization with the handset. For this reason, cordless DECT phones are being criticized for their contribution to the accumulation of electromagnetic pollution and for increasing the concern about their potential in causing health hazards. In fact, that was the main reason we have included this source in our EMF repertoire, exploring the effects on mice (Fragopoulou et al., 2012) and in Drosophila (Margaritis et al., 2013) as well as in this study.

So far, various *in vitro* and *in vivo* studies have shown many effects after exposure of biological material to RF (radio frequencies); DNA breaks (Diem et al., 2005) and apoptosis (Guler et al., 2011), alterations in gene expression (Czyz et al., 2004; Pacini et al., 2002) and also in protein expression (Fragopoulou et al., 2012; Nylund & Leszczynski, 2006) and memory impairments (Fragopoulou et al., 2010; Ntzouni et al., 2011, 2012), to mention just a few examples. In addition, a considerable number of reports have focused on the induction of oxidative stress and triggering of the stress response in biological systems after exposure to EMFs.

Oxidative stress in general involves the imbalance of free radicals, which are byproducts of normal metabolism. Aerobic organisms produce energy in mitochondria via the respiratory chain during which reactive oxygen species (ROS), such as $O2\cdot^{-}$, are also produced. ROS can react with macromolecules causing protein conformational changes (Dean et al., 1997; Stadtman, 1992) and also structural alterations as shown recently on calf thymus DNA exposed to EMF (Hekmat et al., 2012) at very low E-field intensity (15 V/m) and SAR value (0.04 W/Kg).

Besides, ROS key molecules that are normally investigated for a possible oxidative stress event involve

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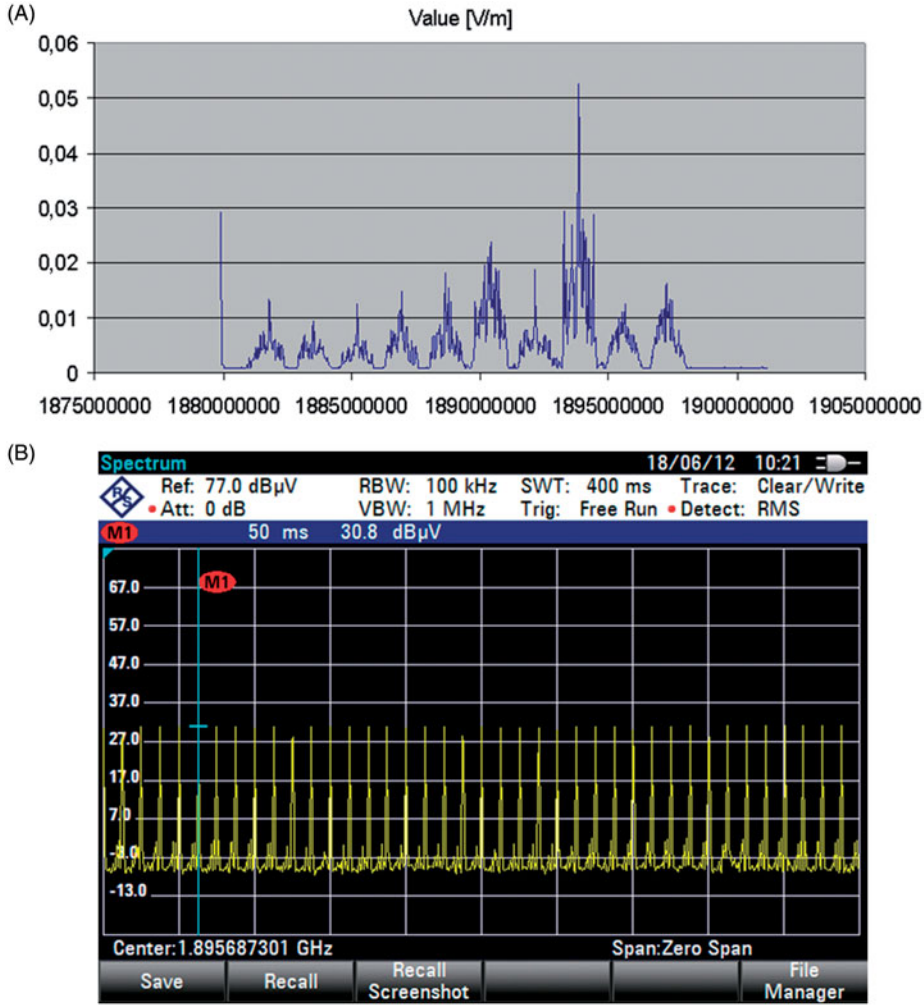


Figure 1. A: The DECT frequency spectrum showing 10 RF channels in the 1880–1900 MHz band. Each channel occupies 2 MHz. (DECT base radiation emission recorded with the NARDA SRM 3000 spectrum analyzer). B: Wireless DECT base emission under zero span and 400 ms sweep rate, showing the 100 Hz repeat rate (40 peaks for 400 ms = 10 ms repeat rate which corresponds to 100 Hz). (Recorded with Rohde & Schwarz FSH8 spectrum analyzer).

malon-di-aldehyde (MDA) implicated in lipid peroxidation, catalase (CAT) breaking down hydrogen peroxide and the specific antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px).

A large number of publications have studied the possible link between oxidative stress and EMF exposure using various sources, SAR levels and biological systems at exposure carrier frequencies around 900 MHz, Irmak et al. (2002) in rabbits, Ilhan et al. (2004) and Yurekli et al. (2006) in rats' brain as well as in blood tissue and the brain of guinea pigs (Meral et al., 2007). Seyhan's group from Ankara Gazi University has extensively demonstrated the oxidative potential of EMFs in various model systems and under various exposure conditions; Kismali et al. (2009) used a commercial mobile phone having a SAR value of 0.81 W/Kg and exposed guinea pigs for 10 min per day for 7 d; increased MDA levels were found in the plasma. A year later Ozgur et al. (2010), from the same group, using also guinea pigs, showed induction of MDA, nitric oxide (NOx) levels and decrease in GSH-Px in the liver after 10 and 20 min daily exposure for 7 d to a 1800 MHz Global System for Mobile Communications (GSM) modulated signal (SAR value of 0.38 W/Kg). Esmekaya et al. (2011) found oxidative stress induction in heart, lung, testis and liver tissues in male Wistar albino rats (pulse-modulated 900 MHz, SAR 1.2 W/Kg, 20 min/day for 3 weeks). Guler et al. (2010, 2012) used 13-month-old non-pregnant and pregnant New Zealand white rabbits exposed for 15 min per day for 7 d at 1800 MHz (average E-field intensity = 14 V/m) and revealed increased levels of MDA and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in their brain, while same results were detected in the liver of female infants (1 month old) under identical exposure conditions.

Studies on individual cells have shown that exposure to RF radiation, applying different SAR values, can provoke oxidative stress in various cell types. An increase in ROS levels has been reported by Lantow et al. (2006a) in blood cells after exposure to 1.8 GHz RF signal, both continuously and intermittently (SAR = 2 W/Kg). Lens epithelial cells exposed to mobile phone radiation (1.8 GHz for 24 h at a SAR value of 4 W/Kg) reacted by increasing the intracellular ROS levels and causing DNA damage (Wu et al., 2008; Yao et al., 2008).

It is worth mentioning that links between ROS increase and sperm damage through RF exposure have gained research interest as reviewed recently (Kesari et al., 2012). This theory was firstly put forward by Agarwal et al. (2009), who found increased ROS levels concomitant with low sperm motility and viability after irradiation with a mobile phone GSM 850 MHz in talk mode (SAR 1.6 W/Kg) for 60 min. In a similar study, it was shown that exposed human spermatozoa

also produced significantly higher amounts of ROS than background levels after exposure to a continuous wave (CW) signal of 1800 MHz and that the mitochondria were involved in this process (De Iuliis et al., 2009).

However, several studies did not generally reveal any association of ROS/oxidative factors with NIR exposure; Ferreira et al. (2006) irradiated rats with a cellular phone (SAR values between 0.55 and 1.23 W/Kg) during embryogenesis and showed no alterations in any oxidative parameter tested. In fact, cell culture studies have given the most contradictory results suggesting that the effects of EMFs upon oxidative stress may vary depending not only on the exposure protocol but also on the cell type. No effects were observed on some types of blood cells, like Jurkat (IL-2-producing immortalized T lymphocytes) after exposure to a continuous wave (CW) 1950 MHz signal at SAR 0.5 and 2 W/Kg (Brescia et al., 2009) and on lymphocytes and Mono Mac 6 after various types of signals and SAR values after exposure to 1800 MHz radiofrequency radiation (Lantow et al., 2006b; Simkó et al., 2006). In addition, just recently, Hong et al. (2012) using single or multiple frequencies (837 and 1950 MHz) on human MCF10A mammary epithelial cells at a high SAR value of 4 W/Kg for 2 h did not observe any changes either in ROS or in the related oxidant and antioxidant molecules, while Kismali et al. (2012) observed no change in MDA and lipid peroxidation levels in the blood of pregnant and non-pregnant rabbits after exposure to a GSM-like 1800 MHz signal for 15 min per day for 7 d.

In the fruit-fly, the induction of stress by an external stimulus was observed for the first time in early 1960, when larvae of *D. melanogaster* exposed overnight showed, due to incorrect handling at high temperature, a different pattern of gene expression in their salivary gland chromosomes, which led to the discovery of heat shock proteins (HSPs) (Ritossa, 1962). Nowadays, Drosophila is a well-established model organism for studies of development and oxidative stress, not only due to the short-life cycle, but also because its antioxidant enzyme systems are fully characterized and are similar to other vertebrates. The first studies, using Drosophila as a model organism for RF exposure, were more or less simultaneously initiated in our laboratory (Panagopoulos et al., 2000) and that of R. Goodman and M. Blank in Columbia University. In the latter case, Weisbrot et al. (2003) showed a significant increase in hsp70 levels in larvae exposed to emissions from a commercial GSM mobile phone. Subsequently, Lee et al. (2008) observed in Drosophila cells increased levels of intracellular ROS and hsp70 and also activation of ERK (Extracellular signal-Regulated Kinases) and JNK (c-Jun N-terminal Kinases) pathway after exposure of adult flies to 835 MHz at SAR values of 1.6 (highest permissible) and 4 W/Kg (above the limit) for 12 and 18 h continuously.

The objective of this study was to explore at the molecular level the possible mechanism(s) underlying our so far findings that RF radiation has a negative impact on insect's oogenesis and reproductive capacity. Specifically, using various EMF sources including cell phone with pulse modulated carrier frequencies of both 900 and 1800 MHz, wireless DECT phone at 1880–1900 MHz, microwave oven at 2440–2480 MHz, Wi-Fi router at 2440–2480 MHz, Blue tooth device at 2440–2480 MHz, baby monitor at 27 MHz and FM signal at 100 MHz, we have observed a decrease in fecundity and an increase in the number of ovarian apoptotic follicles during oogenesis, mostly in two stages; the germarium and stages 7/8 (middle-oogenesis) (Chavdoula et al., 2010; Margaritis et al., 2013; Skouroliakou et al., 2012). Observation of increased apoptotic follicles at these particular stages has shown that RF/MW electromagnetic radiation is a hazardous environmental factor. Therefore, this study aims in the exploration of the basic mechanism(s) underlying the induction of apoptotic cell death and the decrease in fecundity in a model organism, the fruit-fly *D. melanogaster*. We chose to study the levels of ROS in the units of reproduction, that is, the ovaries of flies subjected to whole-body irradiation by a wireless DECT base radiation having very specific pulsed characteristics at the 1880–1900 MHz frequency band (Figures 1–3) and at moderate power/SAR levels. For comparative purposes, we also investigated ROS levels in the bodies of female and male flies following whole-body DECT exposure under various conditions. After obtaining data demonstrating ROS levels increase by DECT irradiation, we considered of utmost importance to investigate the possibility of recovery mechanisms functioning after stopping the exposure in order to explore the effectiveness of the antioxidant mechanism(s) on a time scale.

Materials and methods

Fly culture

All the experiments were performed with the dipteran flies *D. melanogaster*, Oregon R, wild type. All flies reared on same diet containing agar, rice flour, tomato paste, sugar, yeast and propionic acid. The adults from the stock population were removed from the culture bottles (12 cm height and 6 cm diameter). Newly emerged flies were collected using diethyl ether within 4–6 h of eclosion and maintained at a density of 30 flies per vial (15 males and 15 females per vial of 3 cm diameter and 8 cm height) for 96 h, till the fourth day of their adult life, when they were sacrificed for ROS detection. Four-day-old flies are considered to be sexually and reproductively mature for egg-laying and their ovaries consist of all stages (1-14B) of developmental follicles from germarium to mature egg (stage 14B) (Margaritis, 1986). Control flies were kept at 25 °C in a culture room, totally protected from electromagnetic radiation, with standard 12:12 h light/dark cycle and 50% relative humidity. Sham Exposed and Exposed flies were kept in a separate room but cultured under similar conditions as the Control group, 12:12 h light/dark cycle and 50% relative humidity.

Exposure system

Groups of 4-day-old flies were exposed either shortly for 0.5 and 1 h or for longer periods of 6, 24, or 96 h to a DECT base radiation (Figure 2), which consists of 10 channels of sequential scanning each one having 2 MHz range and pulsed at 100 Hz with a 0.08 pulse duration (Figure 3B). The average E-field value of 2.7 V/m for 6 min, according to ICNIRP (1998), under the allocated band of 1.88–1.90 GHz was measured with the FSH8 Rohde & Schwarz Spectrum Analyzer (Munich, Germany) using the Near Field Probe

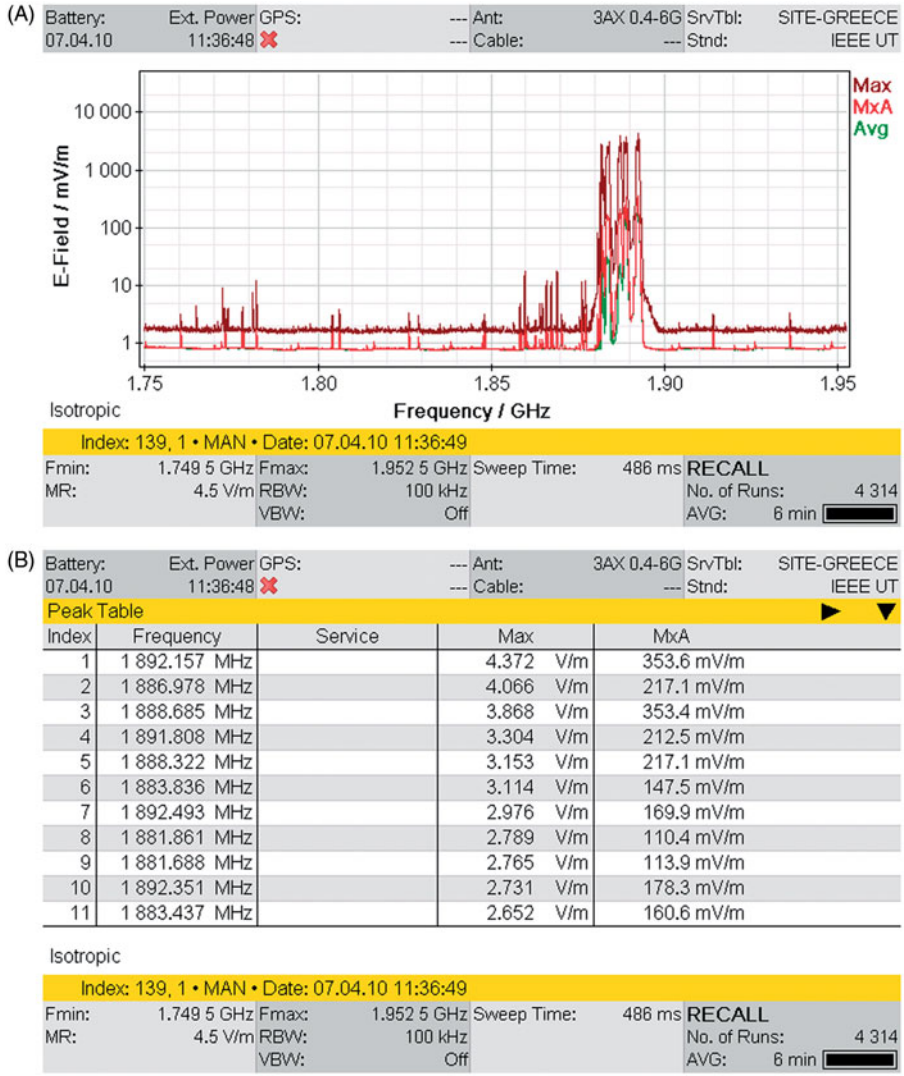


Figure 2. A: DECT base emission at 1880–1900 MHz depicting maximum (Max), maximum average (MxA) and average (Avg) – 6 min electrical field intensity values. B: Dominant frequencies of the spectrum shown in A, according to their average (MxA) and the maximum values (Max). For each frequency there is a nearly 10-fold difference between average and maximum electrical field intensities recorded at a 6 min period. (Spectrum analysis made with NARDA SRM3006).

Set HZ-15. All measurements were made by inserting the probe within an identical fly-culture vial with food as those used for the maintenance and irradiation of the flies. According to the formula $SAR = \sigma E^2/\rho$ and the values of σ and ρ proposed by Lee et al. (2008) for flies (electrical conductivity $(\sigma) = 1.19$ Siemens/m and mass density $(\rho) = 1.000\,kg/m^3$), the SAR value for the measured electrical field intensity of 2.7 V/m was estimated to be 0.009 W/Kg, assuming that the E-field value of 2.7 V/m measured in the air within the vials is the same within the flies. No fantom construction was possible to verify this assumption due to the size of the biological specimen. No exposure was performed having the DECT base in communication with a handset.

The spectrum and pulse characteristics of DECT base idle emission are shown in Figures 1–3. NARDA instruments SRM 3000 and SRM 3006 (Narda Safety solutions, Inc, Germany) and Rohde & Schwarz FSH8 spectrum analyzer were used to record the spectra.

Measuring of ROS Levels

ROS levels were measured using 10 μM of the oxidant-sensitive fluorescent acetyl ester CM-H_2DCFDA (5 -(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate) dissolved in DMSO. CM-H_2DCFDA is a general oxidative stress indicator that can enter cells by penetrating the cell membrane through passive diffusion. Inside the cell its acetate groups are cleaved by intracellular esterases and oxidation by ROS lead to the formation of fluorescent DCF product, which can be detected via fluorometry.

Female and male flies' bodies were prepared after light anesthesia, with diethyl ether, to remove their wings (plus ovaries from the females) and collected in tubes containing 200 μl PBS. The ovaries were removed from the females after dissection in Ringer's solution and were separately analyzed for ROS levels. After their collection, flies' bodies or ovaries were incubated continuously for 30 min with CM-H_2DCFDA at 24 °C in the dark. Then, the ester was removed and incubation followed for 20 min in PBS. Subsequently, samples were washed three times and homogenized in 200 μl 1% NP 40. The quantification of fluorescence was made at the supernatant VersaFluor™ Fluorometer System (Bio-Rad, 170-2402, Hercules, CA) with excitation filter at 490 nm and emission at 520 nm. For the recovery experiments, various time points were tested as trials before finalizing the most promising values. In every set of experiments, duplicated samples were used for the exposed samples and the same run included control and sham-exposed flies. Fifteen bodies and pairs of ovaries from 15 females were used in every sample.

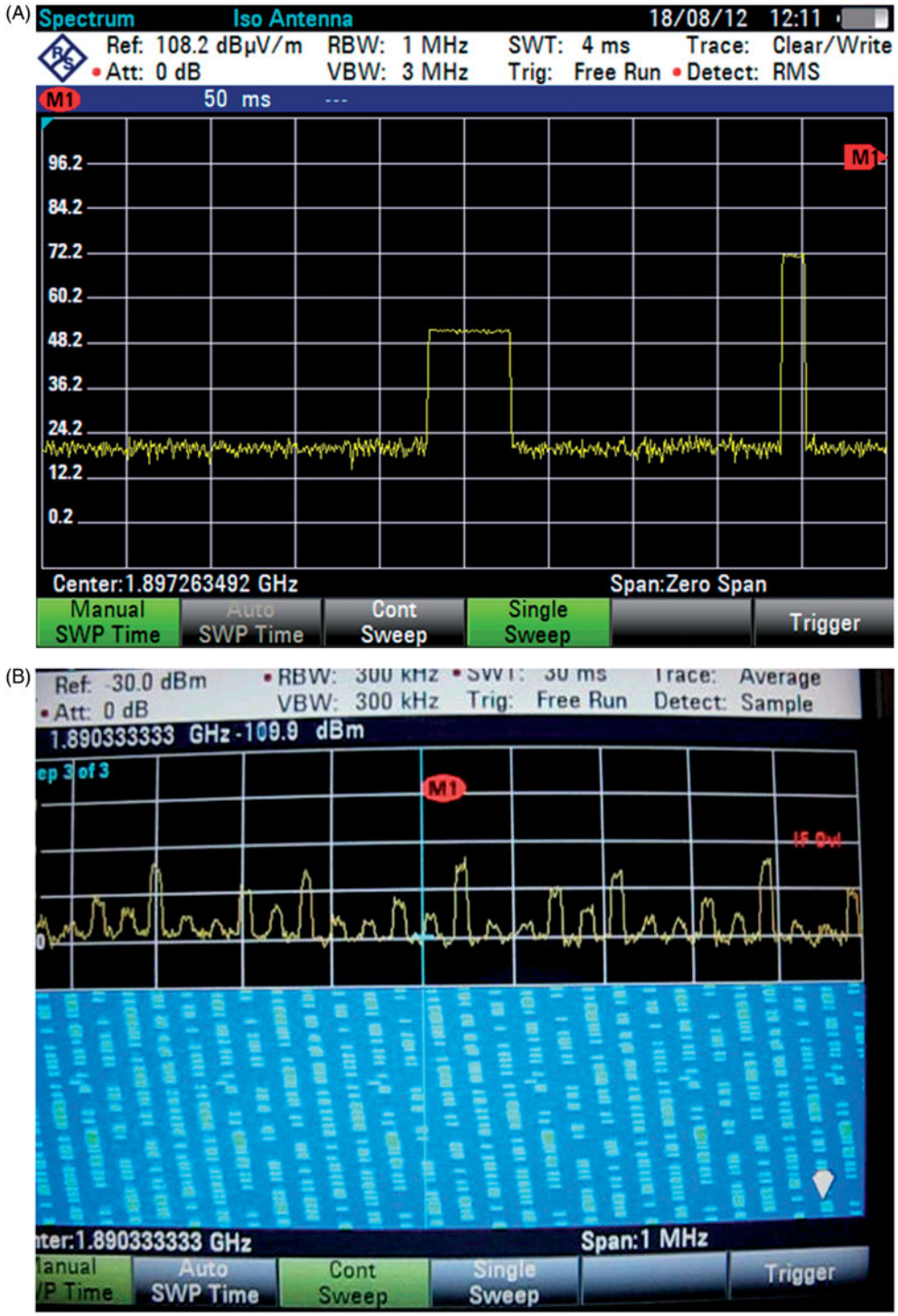


Figure 3. A: Wireless DECT base emission under zero span and 4 ms sweep rate, showing the pulse on the right during idle operation (duration of 0.08 ms) and the pulse on the left during pairing of base and hand set (duration 0.38 ms) (see also Figure 1B). B: 3-D spectrogram demonstrating the discontinuous (pulsed) intensities in DECT base emission profile at a pulse duration at 0.08 ms in just a single frequency of 1890.333 MHz. Horizontal axis (left to right) shows full-scale sweep time (SWT) corresponding to 30 ms as shown in the display on top. Vertical axis on upper half panel represents intensity. The 3rd dimension represented in the lower half panel corresponds to the time scale and shows nearly 30 vertically arranged rows of horizontal lanes the length of each one corresponding to the duration of each pulse (0.08 ms). (Spectrogram recorded with Rohde & Schwarz FSH8 spectrum analyzer).

In total, 307 different samples were measured and were analyzed by SPSS statistics.

Statistical analysis

All data were analyzed by SPSS v.21.0 software (SPSS Inc., Chicago, IL). Differences in mean scores were analyzed using one-way analysis of variance (ANOVA) followed by the LSD post hoc statistics.

Results

To explore if there is any connection between ROS increase and EMF exposure to pulsed radiation deriving from a domestic

wireless DECT apparatus when in idle operation, young adult *D. melanogaster* flies were used as a model system. We chose to investigate this possibility on the male/female bodies and also on the ovarian tissue because we have previously reported induction of apoptotic cell death during oogenesis and reduction of fecundity by various sources of NIR.

Effect of short-term and long-term radiation on ROS levels of *D. melanogaster*

ROS levels in male bodies

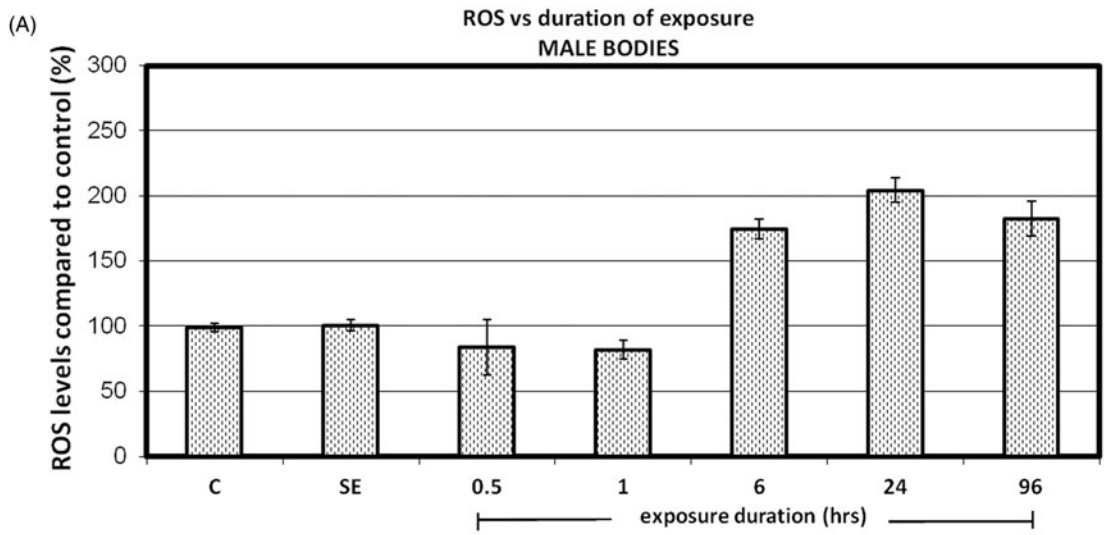
Exposure of newly emerged adult 4-day-old male flies to wireless DECT base radiation for either short-term (0.5 or 1 h) or long-term (6, 24 or 96 h) resulted in a nearly twofold increase in ROS levels at the 6 h sample. Longer exposure (24 and 96 h) provoked no further major alteration in ROS values, whereas short exposure of 30 and 60 min did not raise ROS levels in the male bodies (Table 1, Figure 4). Statistical analysis by one-way ANOVA, LSD post hoc, revealed that ROS levels in male bodies rose gradually and reached a plateau. More specifically, the increase observed after 6 h exposure was statistically significant ($p < 0.001$) compared to control and sham-exposed samples (Figure 4B). The 24 h exposure led to higher levels of ROS, which were statistically significant compared not only to control and sham-exposed flies ($p < 0.001$), but also to the 6 h sample ($p < 0.05$). However, the values recorded at 96 h had no significant difference ($p > 0.05$) with those measured after 6 and 24 h, respectively (Figure 4B), but were of course higher at a statistical significant manner compared to the control and sham exposed samples ($p < 0.001$).

ROS levels in female bodies

Exposure of newly emerged adult 4-day-old female flies to wireless DECT base radiation either for short-term (0.5 or 1 h) or for long-term (6, 24 or 96 h) resulted in a nearly 2.5-fold increase in the bodies' ROS levels at the 24 h sample which was maintained in the flies irradiated for 96 h. Smaller but statistically significant increase ($p < 0.05$)

Table 1. Male bodies. Normalized averaged ROS values of male bodies in percentage, compared to the control values (C) for each experiment, for males sham-exposed flies (SE) and those exposed for 0.5, 1, 6, 24, or 96 h to a wireless DECT base radiation at 2.7 V/m average electrical field intensity (AVG = average, SDV = standard deviation, SER = standard error).

	C	SE	0.5	1	6	24	96
AVG	98.539	100.438	83.609	81.542	174.698	204.248	182.270
SDV	14.703	22.951	42.104	14.453	18.285	26.729	35.125
SER	3.209	4.190	21.052	7.227	7.465	9.450	13.276



(B)

	C	SE	0.5	1	6	24	96
C		0.780	0.179	0.127	0.000	0.000	0.000
SE	0.780		0.118	0.080	0.000	0.000	0.000
0.5	0.179	0.118		0.881	0.000	0.000	0.000
1	0.127	0.080	0.881		0.000	0.000	0.000
6	0.000	0.000	0.000	0.000		0.024	0.569
24	0.000	0.000	0.000	0.000	0.024		0.078
96	0.000	0.000	0.000	0.000	0.569	0.078	

Figure 4. A: Bar graph showing the ROS levels, normalized in percentage compared to the control values for each experiment, in the male bodies of the control (C) and the sham-exposed (SE) flies. The numbers 0.5, 1, 6, 24 and 96 denote hours of exposure of flies to DECT radiation before the ROS assay which was carried out immediately after the end of the exposure. Short exposures of 0.5 and 1 h did not raise the ROS levels but the 6 h sample showed a twofold increase, a value that was slightly raised 18 h later (24 h sample) and remained unaltered 3 d later (96 h exposed flies). B: One-way analysis of variance (ANOVA), LSD statistics comparing all experimental groups revealed statistically significant increased ROS levels at the 6 h exposure ($p < 0.001$). Values of ROS continued to increase from 6 to 24 h ($p = 0.024$), whereas at 96 h the values show no significant difference ($p > 0.05$) compared to those of 6 and 24 h of exposure duration.

was observed after 6 h exposure while short exposure of 30 and 60 min did not alter ROS levels (Table 2, Figure 5). Statistical analysis by one-way ANOVA, LSD post hoc, revealed that ROS levels in female bodies increased gradually from 6 to 96 h and reached a plateau as in the case of the male bodies. ROS levels observed after 6 h exposure increased significantly ($p < 0.001$) compared to control and sham-exposed samples. The 24 and 96 h exposure resulted in a statistically significant increase in ROS levels compared to 6 h ($p < 0.001$), but the increase observed between them was not statistically significant ($p > 0.05$) (Figure 5B).

ROS levels in whole ovaries

Exposure of newly emerged adult 4-day-old female flies to wireless DECT base radiation for short periods of 0.5 and 1 h and for long periods of 6, 24 and 96 h resulted in a nearly 1.5-fold ROS increase ($p < 0.001$) in the ovaries after 0.5 h and a 2.5-fold increase after 1 h exposure (Table 3, Figure 6). It seems that ROS accumulation values peak at a duration of 1 h exposure ($p < 0.001$), and unlike ROS levels recorded from the flies' bodies, these levels were more or less maintained at the 6, 24 and 96 h samples (Figure 7). Statistical analysis by one-way ANOVA, LSD post hoc, revealed that ROS levels in ovaries increased significantly after 0.5 and 1 h radiation compared to those observed in control and sham-exposed samples. Six hours exposure led to statistically significant lower levels compared to those recorded after short-term exposure, while the 24 and 96 h samples showed no further significant alteration in ROS levels ($p > 0.05$) compared to the 6 h sample (Figure 6B).

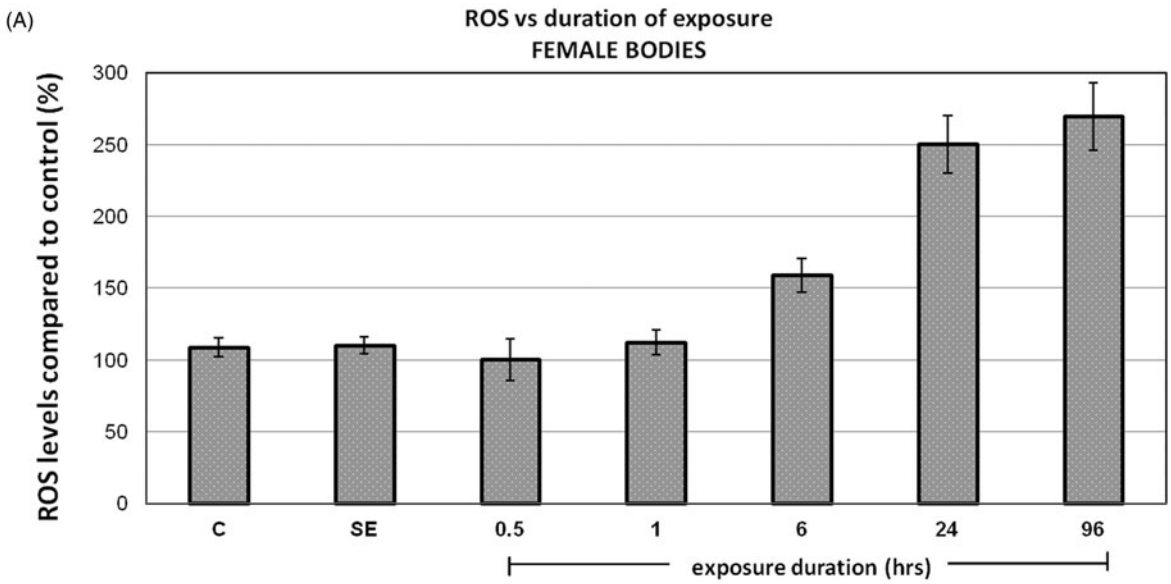
Recovery effect of ROS increase after stopping radiation exposure of flies

To investigate whether there are cellular recovery mechanisms eliminating the observed immediate or gradual ROS increase as a result of wireless DECT base irradiation of *D.*

Table 2. Female bodies. Normalized averaged ROS values of female bodies in percentage compared to the control values (C) for each experiment for female sham-exposed flies (SE) and those exposed for 6, 24 or 96 h to a wireless DECT base radiation at 2.7 V/m average electrical field intensity (AVG = average, SDV = standard deviation, SER = standard error).

	C	SE	0.5	1	6	24	96
AVG	108.796	110.225	100.376	112.179	159.047	250.294	269.794
SDV	22.646	22.877	35.748	24.159	30.5796	48.954	57.367
SER	6.537	5.907	14.594	8.541	11.558	19.986	23.420

(A)



(B)

	C	SE	0.5	1	6	24	96
C		0.886	0.594	0.758	0.000	0.000	0.000
SE	0.886		0.660	0.676	0.000	0.000	0.000
0.5	0.594	0.660		0.485	0.001	0.000	0.000
1	0.758	0.676	0.485		0.005	0.000	0.000
6	0.000	0.000	0.001	0.005		0.000	0.000
24	0.000	0.000	0.000	0.000	0.000		0.282
96	0.000	0.000	0.000	0.000	0.000	0.282	

Figure 5. A: Bar graph showing the ROS level, normalized in percentage compared to the control values for each experiment, in the female bodies of the control (C) and the sham-exposed (SE) flies. The numbers 0.5, 1, 6, 24 and 96 denote hours of exposure to DECT radiation before the ROS assay which was carried out immediately after the end of irradiation. B: One-way analysis of variance (ANOVA), LSD statistics comparing all experimental groups revealed statistical significant ($p < 0.05$) increase of ROS levels in female bodies from 1 to 6 h and from 6 to 24 h but no significant change ($p > 0.05$) from 24 to 96 h of exposure.

melanogaster flies, we measured ROS levels at various time points after stopping the exposure. Before initiation of these experiments, endogenous ROS levels, physiologically existing at the bodies of control or sham-exposed flies, were measured before and after a 24 h period and no change was observed (Figure 8).

ROS recovery in the bodies of 4-day-old exposed flies: 6 h exposure followed by ROS detection immediately, after 1, 4 and 24 h

Having detected that adult flies exposed for 6 h exhibit a statistically significant increase in the levels of ROS (see Figures 4A, 5A), we designed experimental samples with various time points to test for ROS recovery, that is, return of the fluorescence signal to the initiation value before starting the exposure. It was found that a gradual decrease of the elevated ROS values occurs as a function of time in both male and female bodies (Tables 4 and 5, Figures 9 and 10, respectively). In male bodies 1 and 4 h post-exposure period did not decrease ROS levels significantly ($p > 0.05$). However, 24 h without exposure, ROS levels returned to baseline values (Figure 9A) showing a decrease statistically significant ($p < 0.001$) compared to the 6 h exposure value (Figure 9B), dropping down from 174.698 ± 7.465 for the zero time recovery to 101.834 ± 4.102 for the 24 h recovery (Table 4). The same ROS recovery behavior was seen in the female bodies under the same exposure and post-exposure conditions (Figure 10A, B), dropping down from 159.047 ± 12.484 for the zero time recovery to 100.743 ± 9.683 for the 24 h recovery (Table 5).

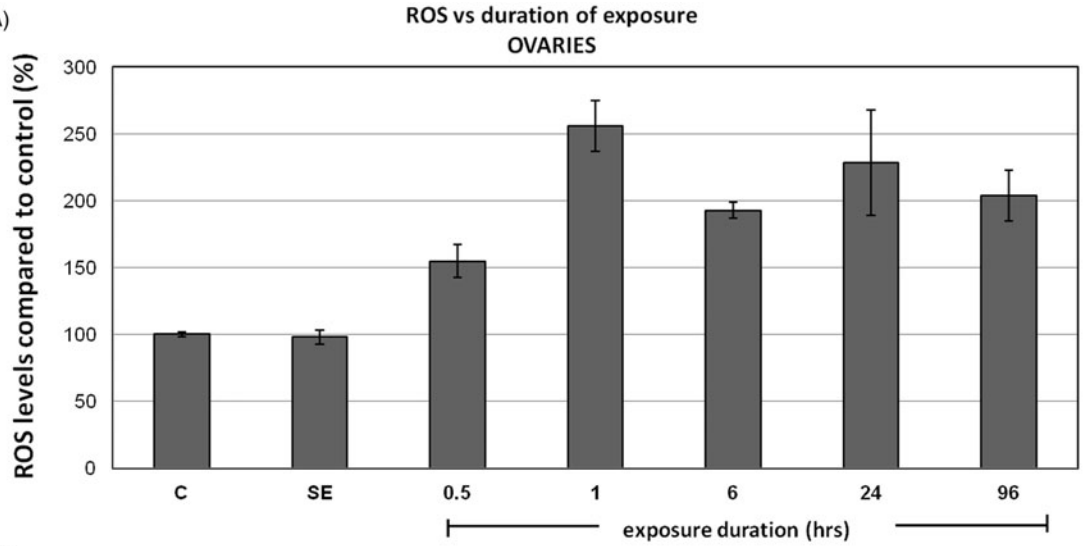
Table 3. Ovaries. Normalized averaged ROS values of ovaries in percentage compared to the control values (C) for each experiment for female sham-exposed flies' ovaries (SE) and those exposed for 0.5, 1, 6, 24 or 96 h to a wireless DECT base radiation at 2.7 V/m average electrical field intensity (AVG = average, SDV = standard deviation, SER = standard error).

	C	SE	0.5	1	6	24	96
AVG	100.305	98.231	155.023	255.836	192.950	228.620	203.756
SDV	7.601	23.974	38.752	56.682	15.013	78.497	46.538
SER	1.900	5.361	12.255	18.894	6.129	39.248	18.999

ROS recovery in the ovaries of 4-day-old exposed flies: 30 or 60 min exposure followed by dissection and ovarian ROS detection either immediately or after 4 h

Having detected that adult flies receiving a single exposure to DECT radiation for 0.5 and 1 h exhibit a statistically significant gradual increase in the levels of ROS of their

(A)



(B)

	C	SE	0.5	1	6	24	96
C		0.863	0.000	0.000	0.000	0.000	0.000
SE	0.863		0.000	0.000	0.000	0.000	0.000
0.5	0.000	0.000		0.000	0.044	0.001	0.010
1	0.000	0.000	0.000		0.001	0.209	0.007
6	0.000	0.000	0.044	0.001		0.126	0.602
24	0.000	0.000	0.001	0.209	0.126		0.284
96	0.000	0.000	0.000	0.007	0.602	0.284	

Figure 6. A: Bar graph showing the ROS level normalized in percentage compared to the control values for each experiment, in the ovaries of the control (C) and the sham-exposed (SE) flies. The numbers 0.5, 1, 6, 24 and 96 denote hours of exposure to DECT radiation before the ROS assay which was carried out immediately after the end of irradiation by dissecting the females and removing the ovaries. B: One-way analysis of variance (ANOVA), LSD statistics comparing all experimental groups revealed statistical significant increased ROS levels in the ovaries at the 0.5, 1, 6, 24, and 96 h exposed females compared to the control and sham-exposed samples ($p < 0.05$). Values of ROS rose after short-term radiation (0.5 and 1 h) having a high value at the 1 h sample ($p < 0.001$) which was more or less maintained in lower levels in the 6, 24 and 96 h exposure of the flies ($p = 0.001$ between 1 and 6 h samples, $p = 0.128$ between 6 and 24 h samples and $p = 0.284$ between 24 and 96 h samples).

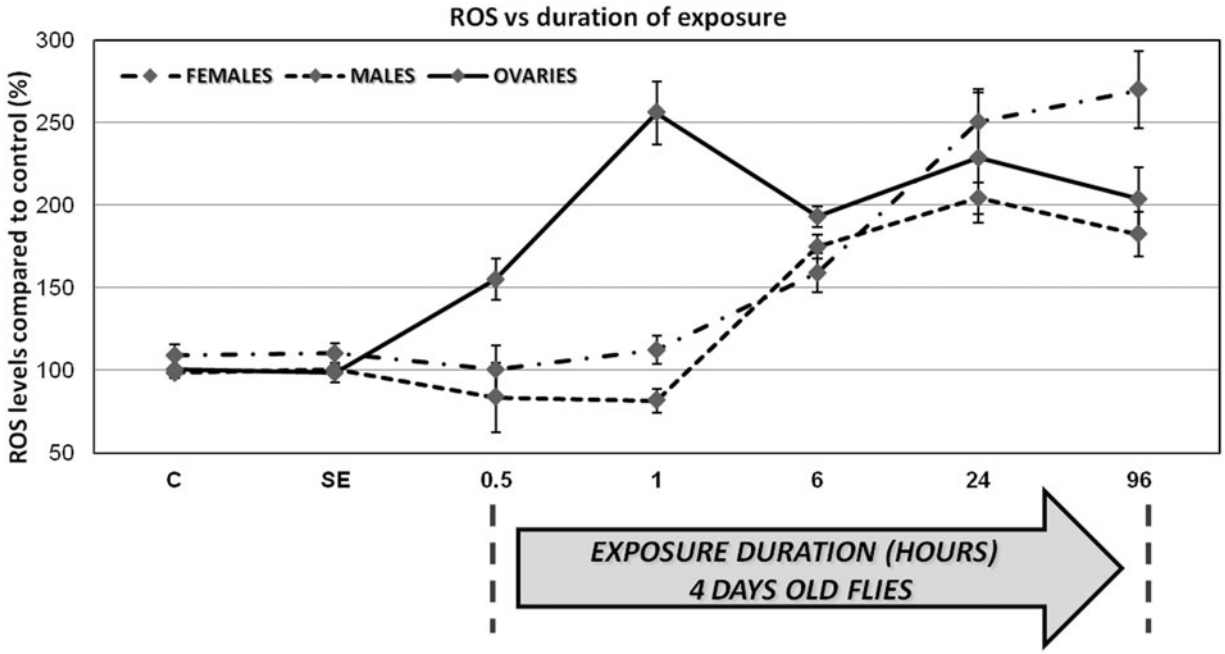
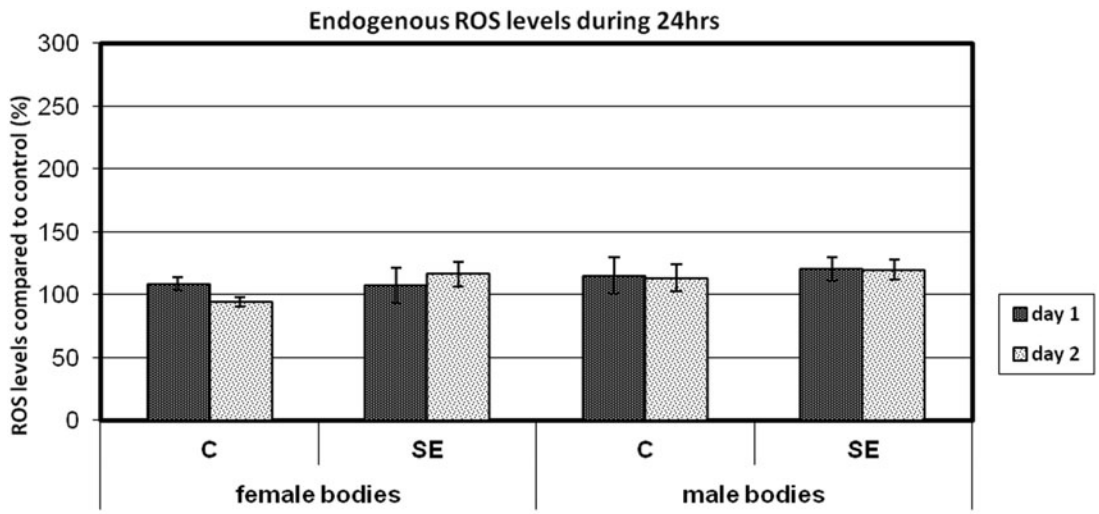


Figure 7. Comparative line-graph showing ROS levels, normalized in percentage compared to the control values, both in the bodies and the ovaries of the flies after various exposure periods. The rapid increase of ROS levels following radiation exposure of 1 h is evident in the ovarian sample.



	female bodies		male bodies	
	C	SE	C	SE
day 1	108.579 ± 4.97	107.557 ± 13.94	115.144 ± 14.33	120.504 ± 9.49
day 2	94.114 ± 3.57	116.393 ± 9.69	113.347 ± 11.12	119.871 ± 7.57

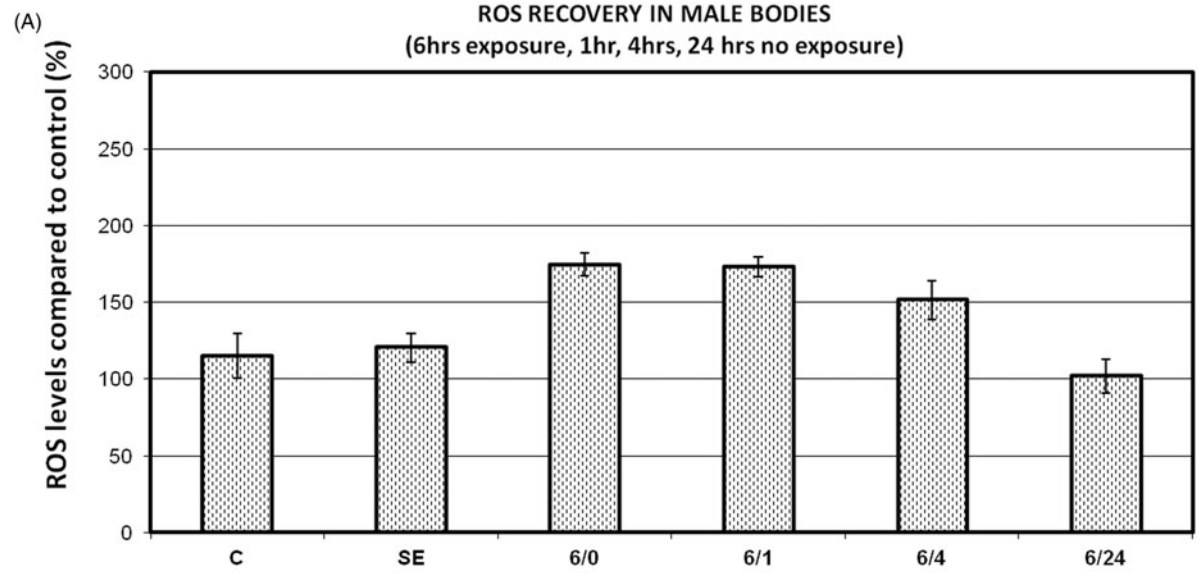
Figure 8. Bar graph showing the endogenous ROS levels, during a 24 h time period, normalized in percentage compared to the control values, in the bodies of the flies (C = control, SE = sham-exposed).

Table 4. ROS recovery males' bodies. Normalized averaged ROS values in percentage compared to the control values (C) for each experiment for male sham-exposed flies (SE) and those exposed for 6 h and then left for 0, 1, 4, 24 h without radiation (AVG = average, SDV = standard deviation, SER = standard error).

	C	SE	6/0	6/1	6/4	6/24
AVG	115.144	120.503	174.698	172.874	151.548	101.834
SDV	40.534	26.853	18.285	17.946	36.026	10.0473
SER	14.331	9.494	7.465	6.345	12.737	4.102

Table 5. ROS recovery females' bodies. Normalized averaged ROS values in percentage compared to the control values (C) for each experiment for female sham-exposed (SE) flies and those exposed for 6 h and then left for 0, 1, 4, 24 h without radiation (AVG = average, SDV = standard deviation, SER = standard error).

	C	SE	6/0	6/1	6/4	6/24
AVG	108.579	107.557	159.047	173.307	160.857	100.743
SDV	12.177	34.135	30.580	14.197	23.697	19.366
SER	4.971	13.935	12.484	6.349	8.378	9.683



(B)

	C	SE	6/0	6/1	6/4	6/24
C		0.705	0.000	0.000	0.014	0.386
SE	0.705		0.001	0.001	0.033	0.227
6/0	0.000	0.001		0.905	0.136	0.000
6/1	0.000	0.001	0.905		0.138	0.000
6/4	0.014	0.033	0.136	0.138		0.002
6/24	0.386	0.227	0.000	0.000	0.002	

Figure 9. A: Bar graph showing ROS recovery levels, normalized in percentage compared to the control values, measured at various time points after the end of the irradiation. Male flies were exposed to a wireless DECT base for 6 h continuously. (C = control, SE = sham-exposed, 6/n = 6 h exposure/n hours after the end of the exposure, n = 0 h, 1 h, 4 h, 24 h). ROS values tend to return to pre-irradiation levels 24 h later. B: One-way analysis of variance (ANOVA), LSD statistics comparing all experimental groups in male bodies at 6 h sample revealed that ROS values tend to decrease gradually with a statistically significant manner ($p < 0.001$) 24 h after the end of the exposure.

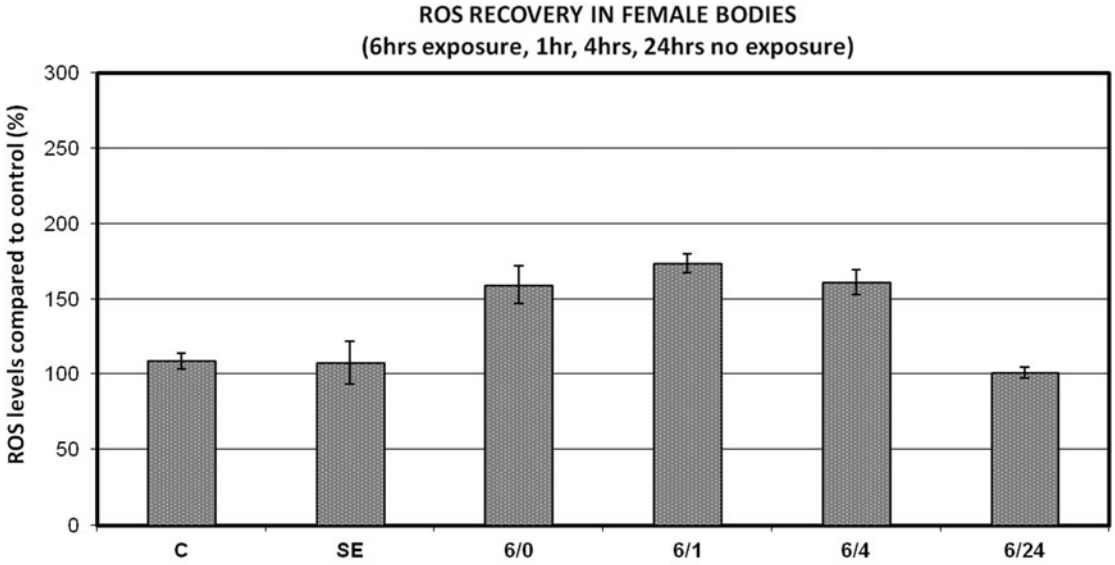
ovaries (see Figure 6A), we designed experimental samples to test for ROS recovery after 4 h post-exposure period. Short (30 or 60 min) exposure of the female flies has an effect on raising the ROS levels immediately which is more pronounce in the 60 min sample (Figure 11A). Follow-up measurement after stopping the exposure for 4 h did reveal that the increased ROS values tend to decrease more considerably and statistically significantly at the 60 min exposure sample ($p = 0.004$) (Figure 11B), dropping down from 253.307 ± 35.274 to 178.484 ± 21.315 (Table 6).

Discussion

The production of ROS is mainly the result of reactions of living organisms in an aerobic environment and the continuous need for oxygen in order for energy to be produced. Superoxide radical ($O2\cdot$) is a physiological byproduct of cells' metabolism and is produced in the respiratory chain via the reduction of molecular oxygen (Squier, 2001). *In vitro* experiments have revealed that 1–3% of the oxygen consumed by mitochondria is converted, in mammals, to hydrogen peroxide (Sastre et al., 2000). However, ROS can be also produced by exogenous factors such as high temperature or UV radiation.

In this study we showed that continuous low-energy pulsed radiofrequency emitted from a wireless DECT base, average E-field density 2.7 V/m and SAR value 0.009 W/Kg calculated according to Lee et al. (2008) increased the levels of ROS in 4-day-old flies of *D. melanogaster*. Both female and male bodies were sensitive to long-term (6, 24 and 96 h) but not to short-term exposure (30 and 60 min), unlike ovaries which showed increased ROS levels already after 30 min of exposure and a peak in ROS values accumulation after 1 h of irradiation. Thus, the organ that seems from this study to have a more severe response to RF radiation is the ovary of the female flies. Sensitivity of the ovary, upon RF radiation, compared to whole body was also demonstrated by Lee et al. (2008). Both bodies and ovaries presented a plateau in ROS levels after certain exposure period. The bodies reached a plateau at 24 h and the levels were maintained at the 96 h of exposure, while in ovaries ROS levels were approximately the same at 6, 24 and 96 h samples. These findings are consistent with the study of Lu et al. (2012) who reported that ROS levels increased in human peripheral blood mononuclear cells (PBMC) after 1, 2 and 4 h exposure to 900 MHz (SAR value 0.4 W/Kg) and reached a maximum value at 6 h and then declined with the passage of irradiation time. The plateau shown in our study at the ROS levels implies possible defensive mechanisms towards the impact of radiation. To further investigate this hypothesis, exposed flies were left for various time points without irradiation. In both male and female

(A)



(B)

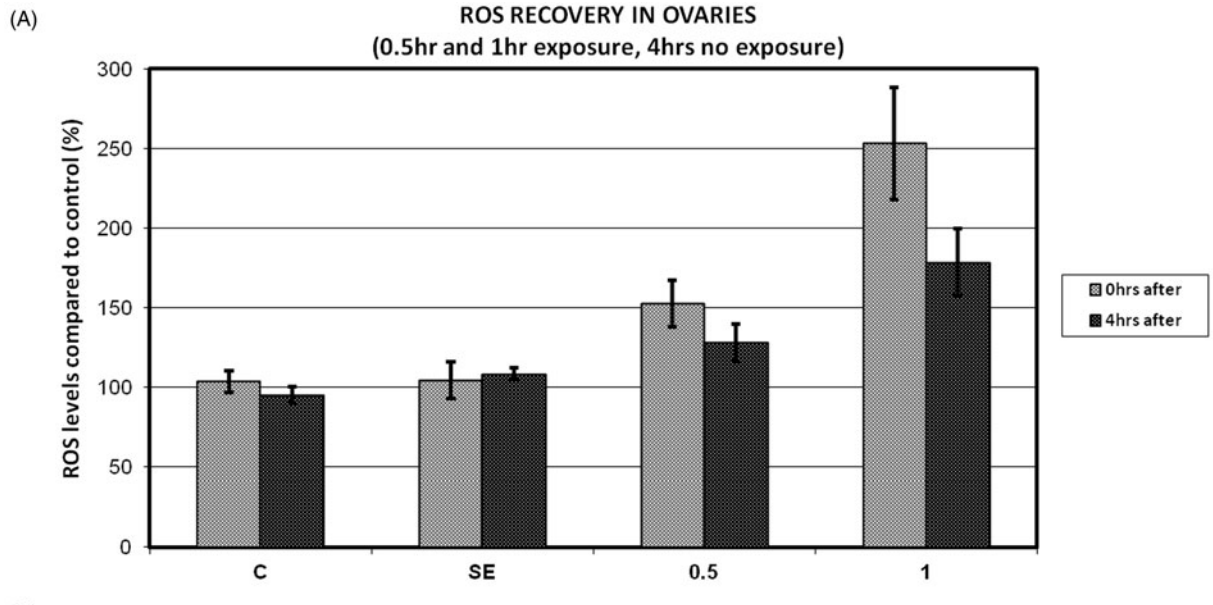
	C	SE	6/0	6/1	6/4	6/24
C		0.943	0.001	0.000	0.000	0.624
SE	0.943		0.001	0.000	0.000	0.670
6/0	0.001	0.001		0.329	0.887	0.001
6/1	0.000	0.000	0.329		0.380	0.000
6/4	0.000	0.000	0.887	0.380		0.000
6/24	0.624	0.670	0.001	0.000	0.000	

Figure 10. A: Bar graph showing ROS recovery levels in female bodies, normalized in percentage compared to the control values for each experiment, measured at various time points after the end of the irradiation. Female flies were exposed to a wireless DECT base continuously for 6 h. (C = control, SE = sham-exposed, 6/n = 6 h exposure/n hours after the end of the exposure, n = 0 h, 1 h, 4 h, 24 h). ROS values tend to return to pre-irradiation levels 24 h later. B: One-way analysis of variance (ANOVA), LSD statistics comparing all experimental groups in female bodies at 6 h sample revealed that ROS values tend to decrease although no statistical significantly till 24 h ($p < 0.001$) after the end of the exposure as was the case in the male bodies (see Figure 9B).

bodies, ROS levels returned to basal levels 24 h after ceasing the exposure. In the ovaries, ROS values tend to return to normal values after 4 h at the 60 min exposure sample. Recent data of our group also showed a recovery phenomenon in mice memory following interruption of mobile phone exposure (Ntzouni et al., 2012). In addition, Franzellitti et al. (2010) exposing human trophoblast HTR-8/SVneo cells for 4, 16 or 24 h with 1.8 GHz, GSM signal have reported DNA damage to be rapidly recovering within 2 h in the absence of irradiation.

So far studies, which are orientated to the hypothesis that nonionizing electromagnetic radiation affects the intracellular redox mechanism and have demonstrated ROS increase, are mainly performed in individual cells, including those of Agarwal et al. (2009) and De Iuliis et al. (2009). These authors detected increase in ROS levels of human spermatozoa after exposure to a cellular telephone in talk mode (SAR 1.46 W/Kg) emitting at 850 MHz frequency and to a CW 1.8 GHz signal, respectively. Wu et al. (2008) and Yao et al. (2008) measured elevated intracellular ROS levels in lens epithelial cells, irradiated for 24 h with a mobile phone 1800 MHz at a SAR value 4 W/Kg, while the same research group (Yao et al., 2008) after exposing the same cell type at a pulse-modulated GSM signal 1.8 GHz (SAR 2, 3 and 4 W/Kg) for 2 h showed also increased ROS levels. Interestingly, the same authors observed that when RF was superposed with 2 μT electromagnetic noise could block RF-induced ROS increase and DNA damage. However, there are also studies with no effect on ROS values using either CW- or pulse-modulated signals; Luukkonen et al. (2010) irradiated neuroblastoma cells (SH-SY5Y) with a CW- or pulse-modulated 872 MHz signal (5 W/Kg), while Poulletier et al. (2011) used an EDGE signal 1800 MHz (SAR 2 and 10 W/Kg) for 1 and 24 h on three human brain cell lines (SH-SY5Y, U87 and CHME5), as well as on primary cortical neuron cultures.

ROS can cause at the cellular level a wide range of responses, from proliferation to cell death. The effect observed depends on the cell type, the intensity and duration of the stimulus. More specifically, low levels of ROS are mitogenic and induce cell proliferation; higher levels cause either permanent or transient cell cycle arrest, while even higher ones lead to cell death through either necrosis or apoptosis (Benhar et al., 2002; Martindale & Holbrook 2002; Rothstein & Lucchesi, 2005). Overproduction of ROS can damage cellular components, mainly lipids in membranes, nucleic acids and proteins, can lead to cell death (Valko et al., 2006) and distortion in spermatozoa in mobile phone-exposed rats (Kesari & Behari, 2012). Lee et al., (2008) showed in parallel with the increased ROS values activation of JNK at a SAR value of 4 W/Kg or ERK pathway (SAR 1.6 W/Kg).



(B)

	C	SE	0.5/0	1/0	0.5/4	1/4
C		0.971	0.052	0.000	0.320	0.004
SE	0.971		0.056	0.000	0.338	0.004
0.5/0	0.052	0.056			0.324	0.296
1/0	0.000	0.000	0.000	0.000	0.000	0.004
0.5/4	0.320	0.338	0.324	0.000		0.046
1/4	0.004	0.004	0.296	0.004	0.046	

Figure 11. A: Bar graph showing ROS levels of the ovaries, normalized in percentage compared to the control values for each experiment. Female flies were exposed to a wireless DECT base for 0.5 or 1 h and their ovaries were tested for ROS levels either immediately or 4 h later. The numbers 0.5/0 and 0.5/4 denote 0.5 h exposure followed by ROS analysis either immediately or 4 h later, respectively. The same holds for the numbers 1/0 and ¼, respectively. ROS levels were also measured in control (C) and sham-exposed (SE) flies under similar timing. B: One-way analysis of variance (ANOVA), LSD statistics comparing all experimental groups revealed a downward trend in ROS values 4 h after interrupting the exposure regardless of its duration. However, statistically significant decrease was observed only in the case of 1 h exposure – 4 h post exposure period ($p = 0.004$).

Table 6. ROS recovery ovaries. Normalized averaged ROS values in percentage compared to the control values (C) for each experiment for sham-exposed (SE) flies' ovaries and those exposed for 0.5 and 1 h and then left for 0 and 4 h without radiation (AVG = average, SDV = standard deviation, SER = standard error).

	C	SE	0.5/0	1/0	0.5/4	1/4
AVG	103.721	104.610	152.619	253.307	128.268	178.464
SDV	16.747	28.432	35.390	86.403	28.266	52.212
SER	6.837	11.607	14.448	35.274	11.540	21.315

Friedman et al. (2007) have also shown in cultured cells induction of ERK cascade within 5 min and a peak at 10–15 min at a power density of 0.005 W/cm^2. These findings imply that cells perceive immediately the electromagnetic radiation as a stress factor and trigger mechanisms, namely ERK cascade mentioned above, to overcome ROS increase and to activate the transcription of genes responsible for their survival. The products of these genes are members of the antioxidant mechanism, including antioxidant enzymes (SOD, CAT, GSH-Px) or heat shock proteins. Such data may explain our recovery results; whereas as reported by Aydin & Akar, (2011), irreversible oxidative damage has been observed after long-term exposure (2 h daily for 45 d) in the major lymphoid organs of rats.

In conclusion, our results indicate that even with very low SAR value ROS activation takes place possibly due to the pulsed and high max value characteristics of the DECT radiation (see Figure 2). Our data, suggestive for a possible recovery mechanism and the plateau observed after continuous exposure, strongly supports the case that an intracellular antioxidant mechanism is induced upon radiation mediated by ROS increase in the bodies of the flies and within the ovaries. However, if the cells cannot overcome the damage caused by ROS, then apoptotic signals are induced. As we have previously shown RF radiation emitted by a commercial cellular phone (Chavdoula et al., 2010), and RF radiation exposure given daily by a DECT base (Margaritis et al., 2013) induces cell death in the check points of oogenesis, that is, developmental stages of germarium and mid-oogenesis (stages 7, 8) and the apoptotic follicles were not created immediately at the end of the exposure but 3–4 h later. The current findings of the immediate ROS increase (within 30–60 min), in relation to the above-mentioned results, indicate that DNA damage, observed after RF exposure, may not be direct but through oxidative stress caused by electromagnetic radiation.

However, oxidative DNA damage and the role of the antioxidant machinery merit further investigation.

Authors contribution

The results presented herein were performed in the laboratory of electromagnetic biology utilizing the established protocol of insect culture and EMF exposure setup. All experiments have independently been replicated and only those fulfilling the statistical criteria have been used. Authors' contribution is as follows: A. K. Manta is a PhD student, performed all experiments on fly culture and ROS assay-spectroscopy and gathered most of the literature, contributed to the design of the experiments, the writing of the manuscript and making the bar-graphs. D. J. Stravopodis contributed to the design of the experiments, the evaluation of the data, the critical reading of the discussion and the revision. I. S. Papassideri contributed to the evaluation of the data and the final revision. L. H. Margaritis, designed and conceived most of the experiments, gathered the data, made the statistics, the EMF measurements and the dosimetry and assembled the final manuscript.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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